Methods of Treating Mesothelioma Using an Antisense Oligonucleotide to Thymidylate Synthase

FIELD OF THE INVENTION

The present invention pertains to the field of cancer therapeutics and in particular to antisense oligonucleotides for the treatment of cancer.

BACKGROUND

A number of proteins have been implicated in cancer and, as a result, are targeted by standard chemotherapeutics for cancer treatment. An example of such a protein is thymidylate synthase (TS), which is an essential enzyme in *de novo* production of thymidylate (Carreras and Santi, 1995, *Annu.Rev.Biochem.* **64**:721-762). Due to the crucial role of TS in DNA synthesis and cell proliferation, it has been an important target for cancer chemotherapy for many years (Danenberg, 1977, *Biochim.Biophys.Acta* **473**:73-92; Danenberg *et al.*, 1999, *Semin.Oncol.* **26**:621-631).

Chemotherapeutics that inhibit TS, such as 5-fluorouracil (5-FU) and its variants, have become integral drugs in standard treatments for colorectal cancer (Papamichael, 1999, *Oncologist.* **4**:478-487). Raltitrexed (Tomudex®) and pemetrexed (Alimta®) are other TS inhibiting chemotherapeutics with a potential role in a range of cancers including mesothelioma. Although reasonably successful in clinical use, these drugs suffer from problems of dose-limiting toxicity and outgrowth of resistant cells, motivating the continued search for alternative treatments.

The use of antisense oligonucleotides as therapeutic molecules is known. Several antisense ODNs targeting a variety of molecules have been shown to have antiproliferative effects against neoplastic cells *in vitro* and *in vivo* (Gewirtz, 2000, *J.Clin.Oncol.* **18**:1809-1811), and several have demonstrated anti-tumour activity and

limited toxicity in Phase I clinical trials (Smith and Wickstrom, 2000, *Methods Enzymol.* **314**:537-580).

Antisense oligonucleotides that target and impact upon the expression of TS mRNA have been described (U.S. Patent No. 6,087,489; International Patent Applications WO 99/15648 and WO 98/49287). A specific antisense oligonucleotide targeting the 3′-untranslated region of TS mRNA has been shown to downregulate TS mRNA and protein and sensitize human HeLa cervical carcinoma and HT-29 colon tumour cells to 5-FU, 5-FUdR and raltitrexed in tissue culture and in immunocompromised mice (Berg *et al.*, 2001, *J.Pharmacol.Exp.Ther.* **298**:477-484). More recently, the use of this antisense oligonucleotide to increase the sensitivity of cells that over-express TS to 5-FUdR has been demonstrated (Ferguson, *et al.,* 2001, *Br. J. Pharmacol.* **134**:1437-1446).

Malignant mesothelioma originates in the mesothelium, *i.e.* the tissue that surrounds the lungs, heart and stomach. The most common form of mesothelioma, affecting approximately three-quarters of mesothelioma patients, is pleural mesothelioma, which affects the lining of the lungs. Approximately 10-20% of patients present with peritoneal mesothelioma, which affects the lining of the abdominal cavity, while pericardial mesothelioma, which affects the lining surrounding the heart, is very rare.

Mesothelioma is an aggressive malignancy and standard treatment for all but localized mesothelioma is generally not curative. Most patients are candidates for chemotherapy at some point in their treatment, but no standard regimen has been established. The most consistent chemotherapeutics for the treatment of mesothelioma include doxorubicin, epirubicin, mitomycin, cyclophosphamide, ifosfamide, cisplatin, carboplatin and antifolates. More recently, the folate-based inhibitor of TS pemetrexed (Alimta®) has demonstrated promising activity against mesothelioma in clinical trials. A Phase III Trail of pemetrexed with cisplatin showed statistically significant improvement of the pemetrexed/cisplatin combination over cisplatin and may result in the use of pemetrexed/cisplatin as first-line standard chemotherapy for malignant pleural mesothelioma (Manegold, *Semin Oncol.* 2003 Aug; 30(4 Suppl 10): 32-6). Furthermore, a similar randomised trial of cisplatin/raltitrexed versus cisplatin

alone showed a borderline significant survival benefit which also establishes cisplatin/raltitrexed as a potential standard of care (EORTC study 08983; Vanmeerbeeck J.P., *et al.*, presented at ASCO 2004). The EMPHACIS clinical trial reported an increase in median survival from 9.3 to 12.1 months in patients treated with cisplatin and pemetrexed versus patients treated with cisplatin alone (Proc ASCO 2002, 21:5a).

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

An object of the present invention is to provide antisense oligonucleotides directed to thymidylate synthase and uses thereof to treat mesothelioma and other cancers. In accordance with an aspect of the present invention, there is provided a method of treating malignant mesothelioma in an animal comprising administering to said animal an effective amount of an antisense oligonucleotide of 7 to about 100 nucleotides in length, said antisense oligonucleotide comprising a sequence of at least 7 consecutive nucleotides complementary to a thymidylate synthase mRNA.

In accordance with another aspect of the invention, there is provided a method of treating malignant mesothelioma in an animal comprising administering to said animal an effective amount of an antisense oligonucleotide of 7 to about 100 nucleotides in length in combination with one or more chemotherapeutic agents, said antisense oligonucleotide comprising a sequence of at least 7 consecutive nucleotides complementary to a thymidylate synthase mRNA.

In accordance with another aspect of the invention, there is provided a method of enhancing the cytotoxity of a chemotherapeutic agent against neoplastic cells comprising the step of contacting said cells with an effective amount of an antisense oligonucleotide of 7 to about 100 nucleotides in length and a chemotherapeutic agent,

said antisense oligonucleotide comprising at least 7 consecutive nucleotides complementary to a thymidylate synthase mRNA.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts a schematic of the location and the nucleotide sequence of an antisense oligonucleotide targeting thymidylate synthase according to one embodiment of the invention, together with the nucleotide sequence of a scrambled control oligonucleotide;

Figure 2A-F illustrates the effect of an antisense oligonucleotide targeting thymidylate synthase according to one embodiment of the invention on *in vitro* proliferation of various tumour cell lines;

Figure 3 illustrates the effect of an antisense oligonucleotide targeting thymidylate synthase (TS) according to one embodiment of the invention on TS mRNA levels in tumour cells *in vitro*;

Figure 4 illustrates the effectiveness of antisense oligonucleotide delivery in HeLa cells. (A) depicts micrographs of cells treated with control scrambled oligonucleotide or a TS antisense oligonucleotide, (B) shows the effect of varying incubation time with the oligonucleotide on delivery and (C) shows the effect of varying the concentration of oligonucleotide on delivery;

Figure 5 compares uptake of antisense oligonucleotide in various tumour cell lines;

Figure 6 illustrates the effect of treatment with thymidylate synthase (TS) antisense oligonucleotide in combination with TS-targeting chemotherapeutic agents on colon cancer (HT-29) cells;

Figure 7A-E illustrates the effect of an antisense oligonucleotide targeting TS according to one embodiment of the invention on *in vitro* proliferation of various tumour cell lines;

Figure 8 illustrates the effect of an antisense oligonucleotide targeting TS according to one embodiment of the invention on TS mRNA levels in mesothelioma 211H, 2052, and H28 cells *in vitro*;

Figure 9 A-C illustrates the quantitation of RT-PCR products from mesothelioma 211H, 2052, and H28 cells treated with an antisense oligonucleotide of the invention targeting TS;

Figure 10A-E illustrates the effect of an antisense oligonucleotide targeting TS according to one embodiment of the invention in combination with pemetrexed (Alimta®) on *in vitro* proliferation of various tumour cell lines;

Figure 11A-D illustrates the effect of an antisense oligonucleotide targeting TS according to one embodiment of the invention in combination with 5-FUdR on *in vitro* proliferation of various tumour cell lines;

Figure 12A-C illustrates the illustrates the effect of an antisense oligonucleotide targeting TS according to one embodiment of the invention in combination with gemcitabine on *in vitro* proliferation of various tumour cell lines; and

Figure 13 provides the sequence of the human thymidylate synthase mRNA (SEQ ID NO:8).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of treating cancer, in particular malignant mesothelioma, with antisense oligonucleotides against the gene encoding a mammalian thymidylate synthase protein. The antisense oligonucleotides of the invention are capable of inhibiting the growth and/or metastasis of cancer cells, including drug resistant cancer cells. The present invention also contemplates that the antisense oligonucleotides may be used as part of a combination therapy in conjunction with one or more chemotherapeutic agents in the treatment of cancer. The antisense oligonucleotides may also be used to sensitize cancer cells, including drug resistant cells, to a chemotherapeutic.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

The terms "antisense oligonucleotide" and "antisense oligodeoxynucleotide" (ODN) as used herein refer to a nucleotide sequence that is complementary to a mRNA for a target gene. In the context of the present invention, the target gene is the gene encoding a mammalian thymidylate synthase protein.

The term "selectively hybridise" as used herein refers to the ability of a nucleic acid to bind detectably and specifically to a second nucleic acid. Oligonucleotides selectively hybridise to target nucleic acid strands under hybridisation and wash conditions that minimise appreciable amounts of detectable binding to non-specific nucleic acids. High stringency conditions can be used to achieve specific hybridization conditions as known in the art. Typically, hybridization and washing are performed at high stringency according to conventional hybridization procedures and employing one or more washing step in a solution comprising 1-3 x SSC, 0.1-1% SDS at 50-70°C for 5-30 minutes.

The term "corresponds to" as used herein with reference to nucleic acid sequences means a polynucleotide sequence that is identical to all or a portion of a reference polynucleotide sequence. In contradistinction, the term "complementary to" is used herein to mean that the polynucleotide sequence is identical to all or a portion of the complement of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used herein to describe the sequence relationships between two or more polynucleotides: "reference sequence," "window of comparison," "sequence identity," "percent sequence identity," and "substantial identity." A "reference sequence" is a defined sequence used as a basis for a sequence

comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA, gene sequence, or may comprise a complete cDNA, gene sequence. Generally, a reference polynucleotide sequence is at least 20 nucleotides in length, and often at least 50 nucleotides in length.

A “window of comparison”, as used herein, refers to a conceptual segment of the reference sequence of at least 15 contiguous nucleotide positions over which a candidate sequence may be compared to the reference sequence and wherein the portion of the candidate sequence in the window of comparison may comprise additions or deletions (*i.e.* gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The present invention contemplates various lengths for the window of comparison, up to and including the full length of either the reference or candidate sequence. Optimal alignment of sequences for aligning a comparison window may be conducted using the local homology algorithm of Smith and Waterman (*Adv. Appl. Math.* (1981) 2:482), the homology alignment algorithm of Needleman and Wunsch (*J. Mol. Biol.* (1970) 48:443), the search for similarity method of Pearson and Lipman (*Proc. Natl. Acad. Sci. (U.S.A.)* (1988) 85:2444), using computerised implementations of these algorithms (such as GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 573 Science Dr., Madison, WI), using publicly available computer software such as ALIGN or Megalign (DNASTAR), or by inspection. The best alignment (*i.e.* resulting in the highest percentage of identity over the comparison window) is then selected.

The term “sequence identity” means that two polynucleotide sequences are identical (*i.e.* on a nucleotide-by-nucleotide basis) over the window of comparison.

The term “percent (%) sequence identity,” as used herein with respect to a reference sequence is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the residues in the reference sequence over the window of comparison after optimal alignment of the sequences and introducing gaps, if

necessary, to achieve the maximum percent sequence identity, without considering any conservative substitutions as part of the sequence identity.

The term "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 50% sequence identity as compared to a reference sequence over the window of comparison. Polynucleotide sequences at least 60% sequence identity, at least 70% sequence identity, at least 80% sequence identity, and at least 90% sequence identity as compared to a reference sequence over the window of comparison are also considered to have substantial identity with the reference sequence.

The terms "therapy," and "treatment," as used interchangeably herein, refer to an intervention performed with the intention of improving a recipient's status. The improvement can be subjective or objective and is related to the amelioration of the symptoms associated with, preventing the development of, or altering the pathology of a disease, disorder or condition being treated. Thus, the terms therapy and treatment are used in the broadest sense, and include the prevention (prophylaxis), moderation, reduction, and curing of a disease, disorder or condition at various stages. Prevention of deterioration of a recipient's status is also encompassed by the term. Those in need of therapy/treatment include those already having the disease, disorder or condition as well as those prone to, or at risk of developing, the disease, disorder or condition and those in whom the disease, disorder or condition is to be prevented.

The term "ameliorate" or "amelioration" includes the arrest, prevention, decrease, or improvement in one or more the symptoms, signs, and features of the disease being treated, both temporary and long-term.

The term "subject" or "patient" as used herein refers to an animal in need of treatment.

The term "animal," as used herein, refers to both human and non-human animals, including, but not limited to, mammals, birds and fish.

Administration of the compounds of the invention "in combination with" one or more further therapeutic agents, is intended to include simultaneous (concurrent)

administration and consecutive administration. Consecutive administration is intended to encompass administration of the therapeutic agent(s) and the compound(s) of the invention to the subject in various orders and via various routes.

As used herein, the term "about" refers to a +/-10% variation from the nominal value. It is to be understood that such a variation is always included in any given value provided herein, whether or not it is specifically referred to.

ANTISENSE OLIGONUCLEOTIDES

Selection and Characteristics

The antisense oligonucleotides of the present invention are targeted to a mammalian thymidylate synthase (TS) gene and thus comprise a nucleotide sequence complementary to a region of the mRNA transcribed from the gene. The sequences of various mammalian TS genes and mRNAs are known in the art and can be readily obtained from Genbank (maintained by the National Center for Biotechnology Information). In one embodiment of the invention, the antisense oligonucleotides are targeted to the human TS gene. In another embodiment, the antisense oligonucleotides comprise a sequence complementary to a portion of the human TS mRNA. The sequence for human TS mRNA can be accessed from GenBank under Accession No. X02308 and is provided herein as Figure 13 (SEQ ID NO:8).

In targeting the antisense to the TS gene or mRNA a determination is made of a site or sites within this gene or its mRNA for the antisense interaction to occur such that the desired effect, *i.e.* modulation of expression of the protein encoded by the gene, will result. Once the target site or sites have been identified, oligonucleotides are chosen that are sufficiently complementary (*i.e.* hybridise with sufficient strength and specificity) to the target to give the desired result.

Generally, antisense oligonucleotides can be targeted to the 5′ untranslated region (5′-UTR), the translation initiation or start codon region, the open reading frame (ORF), the translation termination or stop codon region or the 3′ untranslated region (3′-UTR) of a gene. In accordance with one embodiment of the present invention, the antisense

oligonucleotide is targeted to part of the 3′ untranslated region (3′-UTR) of the TS gene.

The antisense oligonucleotides in accordance with the present invention are selected from a sequence complementary to the TS gene or mRNA such that the sequence exhibits the least likelihood of forming duplexes, hair-pins, or of containing homooligomer / sequence repeats. The oligonucleotide may further contain a GC clamp. One skilled in the art will appreciate that these properties can be determined qualitatively using various computer modelling programs, for example, the program OLIGO® Primer Analysis Software, Version 5.0 (distributed by National Biosciences, Inc., Plymouth, MN).

It is understood in the art that an antisense oligonucleotide need not have 100% identity with the complement of its target sequence. The antisense oligonucleotides in accordance with the present invention have a sequence that is at least about 75% identical to the complement of target sequence. In one embodiment of the present invention, the antisense oligonucleotides have a sequence that is at least about 90% identical to the complement of the target sequence. In a related embodiment, they have a sequence that is at least about 95% identical to the complement of target sequence, allowing for gaps or mismatches of several bases. Identity can be determined, for example, by using the BLASTN program of the University of Wisconsin Computer Group (GCG) software or provided on the NCBI website.

In accordance with the present invention, antisense oligonucleotides comprise at least 7 consecutive nucleotides complementary to a part of a mammalian TS gene and are typically between 7 and 100 nucleotides in length. In one embodiment, the antisense oligonucleotides are between about 7 to about 50 nucleotides in length. In another embodiment, the antisense oligonucleotides are between about 7 to about 35 nucleotides in length. In a further embodiment, the antisense oligonucleotides are between about 15 to about 25 nucleotides in length.

Exemplary, non-limiting examples of sequences for antisense oligonucleotides against a human TS gene are provided in Table 1. In one embodiment of the present invention, the antisense oligonucleotides comprise at least 7 consecutive nucleotides

of the sequence as set forth in any one of SEQ ID NOs: 1, 2, 3, 4, 5 or 6. In another embodiment, the antisense oligonucleotides comprise at least 10 consecutive nucleotides of the sequence as set forth in any one of SEQ ID NOs: 1, 2, 3, 4, 5 or 6.

Table 1. Exemplary Antisense ODNs against Human TS

Sequence (5′ → 3′)	***Complementary Region in TS mRNA***	***SEQ ID NO***
GCCAGTGGCAACATCCTTAA	1184-1203	1
TTGGATGCGGATTGTACCCT	1002-1021	2
ACTCAGCTCCCTCAGATTTG	1436-1455	3
CCAGCCCAACCCCTAAAGAC	1081-1100	4
GGCATCCCAGATTTTCACTC	419-438	5
AGCATTTGTGGATCCCTTGA	380-399	6

The term "antisense oligonucleotides" as used herein includes other oligomeric antisense compounds, including oligonucleotide mimetics, modified oligonucleotides, and chimeric antisense compounds. Chimeric antisense compounds are antisense compounds that contain two or more chemically distinct regions, each made up of at least one monomer unit.

Thus, in the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), or RNA or DNA mimetics. This term, therefore, includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

As is known in the art, a nucleoside is a base-sugar combination and a nucleotide is a nucleoside that further includes a phosphate group covalently linked to the sugar portion of the nucleoside. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound, with the normal linkage or backbone of RNA and DNA being a 3' to 5' phosphodiester linkage. Specific examples of antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include both those that retain a phosphorus atom in the backbone and those that lack a phosphorus atom in the backbone. For the purposes of the present invention, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleotides.

Exemplary modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

In one embodiment of the present invention, the antisense oligonucleotide comprises at least one phosphorothioate linkage. In another embodiment, the antisense oligonucleotide comprises phosphorothioate internucleotide linkages that link the four, five or six 3'-terminal nucleotides of the oligonucleotide. In a further embodiment, the antisense oligonucleotide comprises phosphorothioate internucleotide linkages that link all the nucleotides of the oligonucleotide.

Exemplary modified oligonucleotide backbones that do not include a phosphorus atom are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. Such backbones include morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulphone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulphamate backbones; methyleneimino and methylenehydrazino backbones; sulphonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

The present invention also contemplates oligonucleotide mimetics in which both the sugar and the internucleoside linkage of the nucleotide units are replaced with novel groups. The base units are maintained for hybridisation with an appropriate nucleic acid target compound. An example of such an oligonucleotide mimetic, which has been shown to have excellent hybridisation properties, is a peptide nucleic acid (PNA) [Nielsen *et al.*, *Science*, **254**:1497-1500 (1991)]. In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza-nitrogen atoms of the amide portion of the backbone.

The present invention also contemplates oligonucleotides comprising "locked nucleic acids" (LNAs), which are novel conformationally restricted oligonucleotide analogues containing a methylene bridge that connects the 2′-O of ribose with the 4′-C (see, Singh *et al., Chem. Commun.*, 1998, 4:455-456). LNA and LNA analogues display very high duplex thermal stabilities with complementary DNA and RNA, stability towards 3′-exonuclease degradation, and good solubility properties. Synthesis of the LNA analogues of adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil, their oligomerization, and nucleic acid recognition properties have been described (see Koshkin *et al., Tetrahedron,* 1998, 54:3607-3630). Studies of mis-matched sequences show that LNA obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands.

Antisense oligonucleotides containing LNAs have been described (Wahlestedt *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97:5633-5638), which were efficacious and non-toxic. In addition, the LNA/DNA copolymers were not degraded readily in blood serum and cell extracts.

LNAs form duplexes with complementary DNA or RNA or with complementary LNA, with high thermal affinities. The universality of LNA-mediated hybridization has been emphasized by the formation of exceedingly stable LNA:LNA duplexes (Koshkin *et al., J. Am. Chem. Soc.,* 1998, 120:13252-13253). LNA:LNA hybridization was shown to be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of three LNA monomers (T or A) resulted in significantly increased melting points toward DNA complements.

Synthesis of 2′-amino-LNA (Singh et al., J. Org. Chem., 1998, 63, 10035-10039) and 2'-methylamino-LNA has been described and thermal stability of their duplexes with complementary RNA and DNA strands reported. Preparation of phosphorothioate-LNA and 2′-thio-LNA have also been described (Kumar *et al., Bioorg. Med. Chem. Lett.,* 1998, 8:2219-2222).

Modified oligonucleotides may also contain one or more substituted sugar moieties. For example, oligonucleotides may comprise sugars with one of the following substituents at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Examples of such groups are: $O[(CH_2)_n O]_m CH_3$, $O(CH_2)_n OCH_3$, $O(CH_2)_n NH_2$, $O(CH_2)_n CH_3$, $O(CH_2)_n ONH_2$, and $O(CH_2)_n ON[(CH_2)_n CH_3)]_2$, where n and m are from 1 to about 10. Alternatively, the oligonucleotides may comprise one of the following substituents at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, $SO_2 CH_3$, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an

oligonucleotide, and other substituents having similar properties. Specific examples include 2'-methoxyethoxy (2'-O--CH_2 CH_2 OCH_3, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) [Martin *et al.*, *Helv. Chim. Acta,* **78**:486-504(1995)], 2'-dimethylaminooxyethoxy ($O(CH_2)_2$ $ON(CH_3)_2$ group, also known as 2'-DMAOE), 2'-methoxy (2'-O--CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2) and 2'-fluoro (2'-F).

In one embodiment of the present invention, the antisense oligonucleotide comprises at least one nucleotide comprising a substituted sugar moiety. In another embodiment, the antisense oligonucleotide comprises at least one 2'-O-(2-methoxyethyl) or 2'-MOE modified nucleotide.

Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Oligonucleotides may also include modifications or substitutions to the nucleobase. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5- hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808; The Concise Encyclopedia Of Polymer Science And Engineering, (1990) pp 858-859, Kroschwitz, J. I., ed. John Wiley & Sons; Englisch *et al.*, *Angewandte Chemie, Int. Ed.,* **30**:613

(1991); and Sanghvi, Y. S., (1993) *Antisense Research and Applications*, pp 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C [Sanghvi, Y. S., (1993) *Antisense Research and Applications*, pp 276-278, Crooke, S. T. and Lebleu, B., ed., CRC Press, Boca Raton].

Another oligonucleotide modification included in the present invention is the chemically linkage to the oligonucleotide of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include, but are not limited to, lipid moieties such as a cholesterol moiety [Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA,* **86**:6553-6556 (1989)], cholic acid [Manoharan *et al.*, *Bioorg. Med. Chem. Let.,* **4**:1053-1060 (1994)], a thioether, *e.g.* hexyl-S-tritylthiol [Manoharan *et al.*, *Ann. N.Y. Acad. Sci.,* **660**:306-309 (1992); Manoharan *et al.*, *Bioorg. Med. Chem. Lett.,* **3**:2765-2770 (1993)], a thiocholesterol [Oberhauser *et al.*, *Nucl. Acids Res.,* **20**:533-538 (1992)], an aliphatic chain, *e.g.* dodecandiol or undecyl residues [Saison-Behmoaras *et al.*, *EMBO J.,* **10**:1111-1118 (1991); Kabanov *et al.*, *FEBS Lett.,* **259**:327-330 (1990); Svinarchuk *et al.*, *Biochimie,* **75**:49-54 (1993)], a phospholipid, *e.g.* di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate [Manoharan *et al.*, *Tetrahedron Lett.,* **36**:3651-3654 (1995); Shea *et al.*, *Nucl. Acids Res.,* **18**:3777-3783 (1990)], a polyamine or a polyethylene glycol chain [Manoharan *et al.*, *Nucleosides & Nucleotides,* **14**:969-973 (1995)], or adamantane acetic acid [Manoharan *et al.*, *Tetrahedron Lett.,* **36**:3651-3654 (1995)], a palmityl moiety [Mishra *et al.*, *Biochim. Biophys. Acta,* **1264**:229-237 (1995)], or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety [Crooke *et al.*, *J. Pharmacol. Exp. Ther.,* **277**:923-937 (1996)].

One skilled in the art will recognise that it is not necessary for all positions in a given oligonucleotide to be uniformly modified. The present invention, therefore,

contemplates the incorporation of more than one of the aforementioned modifications into a single oligonucleotide or even at a single nucleoside within the oligonucleotide. The present invention thus further includes antisense compounds that are chimeric compounds. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridising to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridisation techniques known in the art.

In one embodiment of the present invention, the antisense oligonucleotides comprise a phosphorothioate backbone in combination with at least one 2′-MOE modified sugar. In another embodiment, the antisense oligonucleotides comprise a phosphorothioate backbone in combination with one or more 2′-MOE modified sugars at the 3′ and 5′ ends of the oligonucleotide.

In the context of the present invention, an antisense oligonucleotide is "nuclease resistant" when it has either been modified such that it is not susceptible to degradation by DNA and RNA nucleases or alternatively has been placed in a delivery vehicle which in itself protects the oligonucleotide from DNA or RNA nucleases. Nuclease resistant oligonucleotides include, for example, methyl phosphonates, phosphorothioates, phosphorodithioates, phosphotriesters, and morpholino oligomers. Suitable delivery vehicles for conferring nuclease resistance include, for example, liposomes. In one embodiment of the present invention, the antisense oligonucleotides are nuclease resistant.

The present invention further contemplates antisense oligonucleotides that contain groups for improving the pharmacokinetic properties of the oligonucleotide, or groups for improving the pharmacodynamic properties of the oligonucleotide.

Short Interfering RNA (siRNA) Molecules

The present invention further contemplates that the antisense oligonucleotides may be in the form of siRNA molecules. RNA interference mediated by double-stranded siRNA molecules, which are generated in nature when long double-stranded RNA molecules are cleaved by the action of an endogenous ribonuclease, is known in the art to play an important role in post-transcriptional gene silencing [Zamore, *Nature Struc. Biol.,* **8**:746-750 (2001)]. Transfection of mammalian cells with synthetic siRNA molecules having a sequence identical to a target gene has been demonstrated to result in a reduction in the mRNA levels of the target gene [see, for example, Elbashir, *et al., Nature*, **411**:494–498 (2001)]. siRNA molecules are typically 21–22 base pairs in length.

The specificity of siRNA molecules is determined by the binding of the antisense strand of the molecule to its target mRNA. Thus, the antisense oligonucleotides of the present invention can be provided as siRNA molecules which are targeted to a TS gene. As is known in the art, effective siRNA molecules should be less than 30 to 35 base pairs in length to prevent them triggering non-specific RNA interference pathways in the cell via the interferon response. Thus, in one embodiment of the present invention, the siRNA molecules are between about 15 and about 25 base pairs in length. In a related embodiment, they are between 19 and 22 base pairs in length.

The double-stranded siRNA molecules can further comprise poly-T or poly-U overhangs at each end to minimise RNase-mediated degradation of the molecules. In another embodiment of the present invention, the siRNA molecules comprise overhangs at the 3′ and 5′ ends which consist of two thymidine or two uridine residues. Design and construction of siRNA molecules is known in the art [see, for example, Elbashir, *et al., Nature*, **411**:494–498 (2001); Bitko and Barik, *BMC Microbiol.,* **1**:34 (2001)]. In addition, kits that provide a rapid and efficient means of

constructing siRNA molecules by *in vitro* transcription are also commercially available (Ambion, Austin, TX; New England Biolabs, Beverly, MA).

Single-stranded siRNA and short-hairpin siRNA (shRNA) molecules are also known in the art. The present invention contemplates that the antisense oligonucleotides against TS can be provided as single-stranded siRNA molecules and as shRNA molecules.

Preparation of the Antisense Oligonucleotides

The antisense oligonucleotides of the present invention can be prepared by conventional techniques well-known to those skilled in the art. For example, the oligonucleotides can be prepared using solid-phase synthesis using commercially available equipment, such as the equipment available from Applied Biosystems Canada Inc., Mississauga, Canada. As is well-known in the art, modified oligonucleotides, such as phosphorothioates and alkylated derivatives, can also be readily prepared by similar methods.

Alternatively, the antisense oligonucleotides of the present invention can be prepared by enzymatic digestion of the naturally occurring thymidylate synthase gene by methods known in the art.

Antisense oligonucleotides can also be prepared through the use of recombinant methods in which expression vectors comprising nucleic acid sequences that encode the antisense oligonucleotides are expressed in a suitable host cell. Such expression vectors can be readily constructed using procedures known in the art. Examples of suitable vectors include, but are not limited to, plasmids, phagemids, cosmids, bacteriophages, baculoviruses and retroviruses, and DNA viruses. One skilled in the art will understand that selection of the appropriate host cell for expression of the antisense oligonucleotide will be dependent upon the vector chosen. Examples of host cells include, but are not limited to, bacterial, yeast, insect, plant and mammalian cells.

One skilled in the art will also understand that the expression vector may further include regulatory elements, such as transcriptional elements, required for efficient transcription of the antisense oligonucleotide sequences. Examples of regulatory elements that can be incorporated into the vector include, but are not limited to, promoters, enhancers, terminators, and polyadenylation signals. One skilled in the art will appreciate that selection of suitable regulatory elements is dependent on the host cell chosen for expression of the antisense oligonucleotide and that such regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian or insect genes.

In accordance with the present invention, the expression vectors can be introduced into a suitable host cell or tissue by one of a variety of methods known in the art. Such methods can be found generally described in Sambrook *et al.,* 1992; Ausubel *et al.,* 1989; Chang *et al.,* 1995; Vega *et al.,* 1995; and Vectors: A Survey of Molecular Cloning Vectors and Their Uses (1988) and include, for example, stable or transient transfection, lipofection, electroporation, and infection with recombinant viral vectors.

EFFICACY OF THE ANTISENSE OLIGONUCLEOTIDES

1. In vitro *Testing*

Initial determinations of the efficacy of the antisense oligonucleotides of the invention alone, or in combination with one or more chemotherapeutic, can be made using *in vitro* techniques, if required.

For example, the ability of the antisense oligonucleotides to inhibit proliferation of cancer cells can be assessed by culturing cells of a cancer cell line of interest in a suitable medium. After an appropriate incubation time, the cells can be transfected with the antisense oligonucleotide, for example in the presence of a commercial lipid carrier such as lipofectamine, and incubated for a further period of time. Cells are then counted and compared to an appropriate control. Suitable controls include, for example, cells treated with a control oligonucleotide (such as a scrambled form of the test oligonucleotide), cells treated with a standard chemotherapeutic and/or untreated cells.

Alternatively, the antisense oligonucleotides can be tested *in vitro* by determining their ability to inhibit anchorage-independent growth of tumour cells. Anchorage-independent growth is known in the art to be a good indicator of tumourigenicity. In general, anchorage-independent growth is assessed by plating cells from a selected cancer cell-line onto soft agar and determining the number of colonies formed after an appropriate incubation period. Growth of cells treated with the antisense oligonucleotide can then be compared with that of control cells (as described above).

Similar methods can be employed to test the efficacy of the antisense oligonucleotides in combination with chemotherapeutic(s). Suitable controls in this case would include cells treated with the antisense oligonucleotide alone and cells treated with the chemotherapeutic(s) alone.

In one embodiment of the present invention, *in vitro* testing of the antisense oligonucleotides is conducted in a human cancer cell-line. Examples of suitable cancer cell-lines for *in vitro* testing include, but are not limited to, mesothelial cell lines MSTO-211H, NCI-H2052 and NCI-H28, ovarian cancer cell-lines OV90 and SK-OV-3, breast cancer cell-lines MCF-7 and MDA-MB-231, colon cancer cell-lines CaCo, HCT116 and HT29, cervical cancer cell-line HeLa, non-small cell lung carcinoma cell-lines A549 and H1299, pancreatic cancer cell-lines MIA-PaCa-2 and AsPC-1, prostatic cancer-cell line PC-3, bladder cancer cell-line T24, liver cancer cell-lineHepG2, brain cancer cell-line U-87 MG, melanoma cell-line A2058, lung cancer cell-line NCI-H460. Other examples of suitable cell-lines are known in the art.

If necessary, the toxicity of the antisense oligonucleotides can also be initially assessed *in vitro* using standard techniques. For example, human primary fibroblasts can be transfected *in vitro* with the oligonucleotide and then tested at different time points following treatment for their viability using a standard viability assay, such as the trypan-blue exclusion assay. Cells can also be assayed for their ability to synthesize DNA, for example, using a thymidine incorporation assay, and for changes in cell cycle dynamics, for example, using a standard cell sorting assay in conjunction with a fluorocytometer cell sorter (FACS).

2. In vivo *Testing*

The ability of the antisense oligonucleotides to inhibit tumour growth or proliferation *in vivo* can be determined in an appropriate animal model using standard techniques known in the art (see, for example, Enna, *et al., Current Protocols in Pharmacology,* J. Wiley & Sons, Inc., New York, NY).

In general, current animal models for screening anti-tumour compounds are xenograft models, in which a human tumour has been implanted into an animal. Examples of xenograft models of human cancer include, but are not limited to, human solid tumour xenografts in mice, implanted by sub-cutaneous injection and used in tumour growth assays; human solid tumour orthotopic xenografts, implanted directly into the relevant tissue and used in tumour growth assays; human solid tumour isografts in mice, implanted by fat pad injection and used in tumour growth assays; experimental models of lymphoma and leukaemia in mice, used in survival assays, and experimental models of lung metastasis in mice.

For example, the antisense oligonucleotides can be tested *in vivo* on solid tumours using mice that are subcutaneously grafted bilaterally with 30 to 60 mg of a tumour fragment, or implanted with an appropriate number of cancer cells, on day 0. The animals bearing tumours are mixed before being subjected to the various treatments and controls. In the case of treatment of advanced tumours, tumours are allowed to develop to the desired size, animals having insufficiently developed tumours being eliminated. The selected animals are distributed at random to undergo the treatments and controls. Animals not bearing tumours may also be subjected to the same treatments as the tumour-bearing animals in order to be able to dissociate the toxic effect from the specific effect on the tumour. Chemotherapy generally begins from 3 to 22 days after grafting, depending on the type of tumour, and the animals are observed every day. The antisense oligonucleotide of the present invention can be administered to the animals, for example, by i.p. injection or bolus infusion. The different animal groups are weighed about 3 or 4 times a week until the maximum weight loss is attained, after which the groups are weighed at least once a week until the end of the trial.

The tumours are measured after a pre-determined time period, or they can be monitored continuously by measuring about 2 or 3 times a week until the tumour reaches a pre-determined size and / or weight, or until the animal dies if this occurs before the tumour reaches the pre-determined size / weight. The animals are then sacrificed and the tissue histology, size and / or proliferation of the tumour assessed.

Orthotopic xenograft models are an alternative to subcutaneous models and may more accurately reflect the cancer development process. In this model, tumour cells are implanted at the site of the organ of origin and develop internally. Daily evaluation of the size of the tumours is thus more difficult than in a subcutaneous model. A recently developed technique using green fluorescent protein (GFP) expressing tumours in non-invasive whole-body imaging can help to address this issue (Yang and al, *Proc. Nat. Aca. Sci,* (2000), pp 1206-1211). This technique utilises human or murine tumours that stably express very high levels of the *Aqueora vitoria* green fluorescent protein. The GFP expressing tumours can be visualised by means of externally placed video detectors, allowing for monitoring of details of tumour growth, angiogenesis and metastatic spread. Angiogenesis can be measured over time by monitoring the blood vessel density within the tumour(s). The use of this model thus allows for simultaneous monitoring od several features associated with tumour progression and has high preclinical and clinical relevance.

For the study of the effect of the antisense oligonucleotides on leukaemias, the animals are grafted with a particular number of cells, and the anti-tumour activity is determined by the increase in the survival time of the treated mice relative to the controls.

To study the effect of the antisense oligonucleotides of the present invention on tumour metastasis, tumour cells are typically treated with the composition *ex vivo* and then injected into a suitable test animal. The spread of the tumour cells from the site of injection is then monitored over a suitable period of time by standard techniques.

Similar methods can be employed to test the efficacy of the antisense oligonucleotides in combination with chemotherapeutic(s). Suitable controls in this case could include

animals treated with the antisense oligonucleotide alone and animals treated with the chemotherapeutic(s) alone.

In vivo toxic effects of the oligonucleotides can be evaluated by measuring their effect on animal body weight during treatment and by performing haematological profiles and liver enzyme analysis after the animal has been sacrificed.

Table 2: Examples of xenograft models of human cancer

Cancer Model	Cell Type
Tumour Growth Assay Human solid tumour xenografts in mice (sub-cutaneous injection)	Mesothelioma (NCI-H2052) Prostate (PC-3, DU145) Breast (MDA-MB-231, MVB-9) Colon (HT-29) Lung (NCI-H460, NCI-H209) Pancreatic (ASPC-1, SU86.86) Pancreatic: drug resistant (BxPC-3) Skin (A2058, C8161) Cervical (SIHA, HeLa-S3) Cervical: drug resistant (HeLa S3-HU-resistance) Liver (HepG2) Brain (U87-MG) Renal (Caki-1, A498) Ovary (SK-OV-3)
Tumour Growth Assay Human solid tumour isografts in mice (fat pad injection)	Breast: drug resistant (MDA-CDDP-S4, MDA-MB435-To.1)
Survival Assay Experimental model of lymphoma and leukaemia in mice	Human: Burkitts lymphoma (Non-Hodgkin's) (raji) Murine: erythroleukemia (CB7 Friend retrovirus-induced)
Experimental model of lung metastasis in mice	Human: melanoma (C8161) Murine: fibrosarcoma (R3)

3. Efficacy of combinations of the antisense oligonucleotide and one or more chemotherapeutic

As indicated above, the antisense oligonucleotides of the invention can be administered in combination with one or more chemotherapeutic agent. The efficacy of the combination therapy can be tested *in vitro* and *in vivo* as indicated above.

In one embodiment of the present invention, the combination of the antisense oligonucleotide and the chemotherapeutic agent(s) has a greater than additive effect compared to either the antisense oligonucleotide or the chemotherapeutic agent alone. This effect can be measured for example by determining the median lethal dose (LD_{50}) for the chemotherapeutic agent alone and in combination with the antisense oligonucleotide. In another embodiment of the invention, the antisense oligonucleotides increase the cytotoxic effects of one or more chemotherapeutic agents. In a further embodiment, the antisense oligonucleotides act as chemopotentiators of one or more chemotherapeutic agents.

In another embodiment, the combination of the antisense oligonucleotide and the chemotherapeutic agent(s) has an improved therapeutic effect over the therapeutic effect of the antisense oligonucleotide or the chemotherapeutic agent alone. The improved therapeutic effect can be manifested, for example, as an increase in the efficacy of the one or more component of the composition in attenuating tumour growth and/or metastasis and/or a decrease or delay in the toxicity phenomena associated with a component of the combination.

An improved therapeutic effect can be measured, for example, by determining whether the combination results in an improved therapeutic index compared to the individual components.

The ratio of the median effective dose (ED_{50}) and the LD_{50} can be used as an indication of the therapeutic index of a compound. The ED_{50} of a drug is the dose required to produce a specified effect in 50% of a test population and the LD_{50} of a drug is the dose that has a lethal effect on 50% of a test population. The LD_{50} is determined in preclinical trials, whereas the ED_{50} can be tested in preclinical or clinical trials. Preclinical trials are conducted using an appropriate animal model, such

as those described above. Alternatively the therapeutic index can be determined based on doses that produce a therapeutic effect and doses that produce a toxic effect (e.g. ED_{90} and LD_{10}, respectively). During clinical studies, the dose, or the concentration (e.g. solution, blood, serum, plasma), of a drug required to produce toxic effects can be compared to the concentration required for the therapeutic effects in the population to evaluate the clinical therapeutic index. Methods of clinical studies to evaluate the clinical therapeutic index are well known to workers skilled in the art.

In one embodiment of the present invention, the antisense oligonucleotide decreases the observed LD_{50} of at least one of the chemotherapeutic agents in the combination. In another embodiment, the antisense oligonucleotide increases the observed ED_{50} of at least one of the chemotherapeutic agents in the combination.

An improved therapeutic effect can also be manifested as therapeutic synergy. A combination manifests therapeutic synergy when it is therapeutically superior to one of the components when used at that component's optimum dose [T. H. Corbett *et al.*, (1982) *Cancer Treatment Reports*, 66, 1187]. To demonstrate the efficacy of a combination, it may be necessary to compare the maximum tolerated dose of the combination with the maximum tolerated dose of each of the separate components in the study in question. This efficacy may be quantified using techniques and equations commonly known to workers skilled in the art. [T. H. Corbett *et al.*, (1977) *Cancer*, 40, 2660.2680; F. M. Schabel *et al.*, (1979) Cancer Drug Development, Part B, Methods in Cancer Research, 17, 3-51, New York, Academic Press Inc.].

The combination, used at its own maximum tolerated dose, in which each of the components will be present at a dose generally not exceeding its maximum tolerated dose, will manifest therapeutic synergy when the efficacy of the combination is greater than the efficacy of the best component when it is administered alone.

PHARMACEUTICAL COMPOSITIONS

The antisense oligonucleotide may be administered as a pharmaceutical composition with an appropriate pharmaceutically physiologically acceptable carrier, diluent, excipient or vehicle.

For the treatment of most types of cancer, the pharmaceutical compositions are formulated for systemic administration. For the treatment of mesothelioma, the pharmaceutical compositions can be formulated for intracavitary adminstration. The term intracavitary includes intraperitoneal, intrapericardial and intrapleural. The pharmaceutical compositions of the present invention may also be formulated for administration orally, topically, parenterally, by inhalation or spray or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques.

Aqueous suspensions contain the active compound in admixture with suitable excipients including, for example, suspending agents, such as sodium carboxymethylcellulose, methyl cellulose, hydropropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents such as a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example, polyoxyethyene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example, hepta-decaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol for example, polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example, polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or *n*-propyl *p*-hydroxy-benzoate, one or more colouring agents, one or more flavouring agents or one or more sweetening agents, such as sucrose or saccharin.

The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to known art using suitable dispersing or wetting agents and suspending agents such as those mentioned above. The sterile injectable preparation may also be sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Acceptable vehicles and solvents that may

be employed include, but are not limited to, water, Ringer's solution, lactated Ringer's solution and isotonic sodium chloride solution. Other examples are, sterile, fixed oils which are conventionally employed as a solvent or suspending medium, and a variety of bland fixed oils including, for example, synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The pharmaceutical compositions may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to methods known to the art for the manufacture of pharmaceutical compositions and may contain one or more agents selected from the group of sweetening agents, flavouring agents, colouring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with suitable non-toxic pharmaceutically acceptable excipients including, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, such as corn starch, or alginic acid; binding agents, such as starch, gelatine or acacia, and lubricating agents, such as magnesium stearate, stearic acid or talc. The tablets can be uncoated, or they may be coated by known techniques in order to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate may be employed.

Pharmaceutical compositions for oral use may also be presented as hard gelatine capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatine capsules wherein the active ingredient is mixed with water or an oil medium such as peanut oil, liquid paraffin or olive oil.

Oily suspensions may be formulated by suspending the active ingredients in a vegetable oil, for example, arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening

agent, for example, beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and/or flavouring agents may be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active compound in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavouring and colouring agents, may also be present.

Pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oil phase may be a vegetable oil, for example, olive oil or arachis oil, or a mineral oil, for example, liquid paraffin, or it may be a mixtures of these oils. Suitable emulsifying agents may be naturally-occurring gums, for example, gum acacia or gum tragacanth; naturally-occurring phosphatides, for example, soy bean, lecithin; or esters or partial esters derived from fatty acids and hexitol, anhydrides, for example, sorbitan monoleate, and condensation products of the said partial esters with ethylene oxide, for example, polyoxyethylene sorbitan monoleate. The emulsions may also contain sweetening and flavouring agents.

Syrups and elixirs may be formulated with sweetening agents, for example, glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative, and/or flavouring and colouring agents.

Other pharmaceutical compositions and methods of preparing pharmaceutical compositions are known in the art and are described, for example, in "*Remington: The Science and Practice of Pharmacy,*" Gennaro, A., Lippincott, Williams & Wilkins, Philidelphia, PA (2000) (formerly "*Remingtons Pharmaceutical Sciences*").

USE OF THE ANTISENSE OLIGONUCLEOTIDES

The antisense oligonucleotides of the present invention can be used in the treatment of a variety of cancers, including drug resistant tumours. The present invention contemplates that one, or a combination of two or more antisense oligonucleotides may be administered to a patient for the treatment of cancer. The present invention further contemplates that the antisense oligonucleotides can be used in combination with one or more chemotherapeutic agents in the treatment of cancer, including drug resistant cancer.

In one embodiment, the antisense oligonucleotides are used to treat or stabilise malignant mesothelioma. In the context of the present invention, malignant mesothelioma includes peritoneal mesothelioma, pleural mesothelioma and pericardial mesothelioma. As is known in the art, malignant mesothelioma can be classified as Stage I, II, III or IV. In Stage I mesothelioma (localized malignant mesothelioma) the cancer is found in the lining of the chest cavity near the lung and heart or in the diaphragm or the lung. Stage II, II and IV mesotheliomas are considered to be advanced cancers. In Stage II mesothelioma the cancer has spread beyond the lining of the chest to lymph nodes in the chest. At Stage III the cancer has spread into the chest wall, center of the chest, heart, through the diaphragm, or abdominal lining, and in some cases into nearby lymph nodes and at Stage IV the cancer has spread to distant organs or tissues. Recurrent malignant mesothelioma is a term used to describe mesothelioma that recurs after it has been treated and may recur in the lining of the chest or abdomen or in another part of the body. The present invention contemplates that the antisense oligonucleotides described herein can be used to treat all Stages of mesothelioma as well as recurrent mesothelioma and drug resistant mesothelioma.

Examples of other cancers which may be may be treated, stabilised, or prevented in accordance with the present invention include, but are not limited to leukaemia, carcinomas, adenocarcinomas, melanomas and sarcomas. Carcinomas, adenocarcinomas and sarcomas are also frequently referred to as "solid tumors," examples of commonly occurring solid tumors include, but are not limited to, cancer

of the brain, breast, cervix, colon, head and neck, kidney, lung, ovary, pancreas, prostate, stomach and uterus, non-small cell lung cancer and colorectal cancer.

The term "leukaemia" refers broadly to progressive, malignant diseases of the blood-forming organs. Leukaemia is typically characterised by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow but can also refer to malignant diseases of other blood cells such as erythroleukaemia, which affects immature red blood cells. Leukaemia is generally clinically classified on the basis of (1) the duration and character of the disease – acute or chronic; (2) the type of cell involved – myeloid (myelogenous), lymphoid (lymphogenous) or monocytic, and (3) the increase or non-increase in the number of abnormal cells in the blood – leukaemic or aleukaemic (subleukaemic). Leukaemia includes, for example, acute nonlymphocytic leukaemia, chronic lymphocytic leukaemia, acute granulocytic leukaemia, chronic granulocytic leukaemia, acute promyelocytic leukaemia, adult T-cell leukaemia, aleukaemic leukaemia, aleukocythemic leukaemia, basophylic leukaemia, blast cell leukaemia, bovine leukaemia, chronic myelocytic leukaemia, leukaemia cutis, embryonal leukaemia, eosinophilic leukaemia, Gross' leukaemia, hairy-cell leukaemia, hemoblastic leukaemia, hemocytoblastic leukaemia, histiocytic leukaemia, stem cell leukaemia, acute monocytic leukaemia, leukopenic leukaemia, lymphatic leukaemia, lymphoblastic leukaemia, lymphocytic leukaemia, lymphogenous leukaemia, lymphoid leukaemia, lymphosarcoma cell leukaemia, mast cell leukaemia, megakaryocytic leukaemia, micromyeloblastic leukaemia, monocytic leukaemia, myeloblastic leukaemia, myelocytic leukaemia, myeloid granulocytic leukaemia, myelomonocytic leukaemia, Naegeli leukaemia, plasma cell leukaemia, plasmacytic leukaemia, promyelocytic leukaemia, Rieder cell leukaemia, Schilling's leukaemia, stem cell leukaemia, subleukaemic leukaemia, and undifferentiated cell leukaemia.

The term "sarcoma" generally refers to a tumour which originates in connective tissue, such as muscle, bone, cartilage or fat, and is made up of a substance like embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar or homogeneous substance. Sarcomas include soft tissue sarcomas, chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma,

myxosarcoma, osteosarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilms' tumour sarcoma, endometrial sarcoma, stromal sarcoma, Ewing's sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, granulocytic sarcoma, Hodgkin's sarcoma, idiopathic multiple pigmented haemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphoma, immunoblastic sarcoma of T-cells, Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, and telangiectaltic sarcoma.

The term "melanoma" is taken to mean a tumour arising from the melanocytic system of the skin and other organs. Melanomas include, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungal melanoma, and superficial spreading melanoma.

The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases. Exemplary carcinomas include, for example, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatosum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colorectal carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiermoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, haematoid

carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypemephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, naspharyngeal carcinoma, oat cell carcinoma, non-small cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, carcinoma scroti, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, and carcinoma villosum.

The term "carcinoma" also encompasses adenocarcinomas. Adenocarcinomas are carcinomas that originate in cells that make organs which have glandular (secretory) properties or that originate in cells that line hollow viscera, such as the gastrointestinal tract or bronchial epithelia. Examples include, but are not limited to, adenocarcinomas of the breast, lung, pancreas and prostate.

Additional cancers encompassed by the present invention include, for example, Hodgkin's Disease, Non-Hodgkin's lymphoma, multiple myeloma, neuroblastoma, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, malignant pancreatic insulanoma, malignant carcinoid, urinary bladder cancer, premalignant skin lesions, gliomas, testicular cancer, thyroid cancer, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, endometrial cancer, adrenal cortical cancer, and medulloblastoma.

In one embodiment of the present invention, the cancer to be treated is selected from the group of: mesothelioma, solid tumours, breast cancer, colon cancer, ovarian cancer, cervical cancer, and metastatic and multidrug resistant versions thereof.

The cancer may be indolent or it may be aggressive. The antisense oligonucleotides can be used to treat refractory cancers, advanced cancers, recurrent cancers and metastatic cancers. One skilled in the art will appreciate that many of these categories may overlap, for example, aggressive cancers are typically also metastatic.

"Aggressive cancer," as used herein, refers to a rapidly growing cancer. One skilled in the art will appreciate that for some cancers, such as breast cancer or prostate cancer the term "aggressive cancer" will refer to an advanced cancer that has relapsed within approximately the earlier two-thirds of the spectrum of relapse times for a given cancer, whereas for other types of cancer, such as small cell lung carcinoma (SCLC) nearly all cases present rapidly growing cancers which are considered to be aggressive. The term can thus cover a subsection of a certain cancer type or it may encompass all of other cancer types. A "refractory" cancer or tumour refers to a cancer or tumour that has not responded to treatment. "Advanced cancer," refers to overt disease in a patient, wherein such overt disease is not amenable to cure by local modalities of treatment, such as surgery or radiotherapy. Advanced disease may refer to a locally advanced cancer or it may refer to metastatic cancer. The term "metastatic cancer" refers to cancer that has spread from one part of the body to another.

Combination Therapy

As indicated above, the present invention also contemplates the use of the antisense oligonucleotides in combination with one or more chemotherapeutic agents. The chemotherapeutic agent can be selected from a wide range of cancer chemotherapeutic agents known in the art, including those that target thymidylate synthase. Combination therapies using combinations of standard cancer chemotherapeutics are also known in the art and may be used in conjunction with the antisense oligonucleotides.

Combinations of the antisense oligonucleotide of the present invention and standard

chemotherapeutics may act to improve the efficacy of the chemotherapeutic and, therefore, can be used to improve standard cancer therapies. This application is particularly important in the treatment of drug-resistant cancers which are not responsive to standard treatment. Drug-resistant cancers can arise, for example, from heterogeneity of tumour cell populations, alterations in response to chemotherapy and increased malignant potential. Such changes are often more pronounced at advanced stages of disease and have, in part, as an underlying cause, changes in genome/message stability. In one embodiment of the invention, the antisense oligonucleotide of the present invention is used in conjunction with one or more chemotherapeutic agent to treat drug resistant tumours, including drug resistant mesothelioma.

In one embodiment of the present invention, the antisense oligonucleotides are used in conjunction with one or more chemotherapeutic agent that targets TS. Examples of suitable TS inhibiting chemotherapeutics include, but are not limited to, the fluoropyrimidine drugs 5-FU, 5-FUdR, capecitabine (an oral form of a pro-drug of 5-FU) and a topical 5-FU cream (Effudex®), as well as the non-fluoropyrimidine drugs raltitrexed, methotrexate and pemetrexed (Alimta®). These chemotherapeutic agents are used alone and in combination in a variety of treatment regimens against various tumours including colorectal, breast, lung and mesothelioma. In one embodiment of the present invention, the antisense oligonucleotides are used in combination with one or more of 5-FU, 5-FUdR, capecitabine, pemetrexed, methotrexate or raltitrexed. In another embodiment, the antisense oligonucleotides are used in combination with 5-FU, 5-FUdR, or pemetrexed.

For the treatment of mesothelioma, the antisense oligonucleotides can be used in combination with doxorubicin, epirubicin, mitomycin, cyclophosphamide, ifosfamide, cisplatin, carboplatin, 5-FU, raltitrexed or pemetrexed (Alimta®), or a combination of these chemotherapeutics, such as pemetrexed/cisplatin. In one embodiment of the present invention, the antisense oligonucleotides are used in combination with one or more chemotherapeutics selected from the group of: doxorubicin, epirubicin, mitomycin, cyclophosphamide, ifosfamide, cisplatin, carboplatin, 5-FU, raltitrexed or pemetrexed (Alimta®) in order to potentiate the effect of the chemotherapeutic(s).

5-FU has been used as chemotherapeutic for many years alone and in conjunction with other chemotherapeutics. The following exemplary therapeutic regimens are provided with the understanding that one skilled in the art would appreciate that they may be applied to the situations where 5-FU is used alone or conjunction with another chemotherapeutic. A first exemplary regimen is the Mayo regimen, wherein 1 cycle consists of 5-FU administered at 425 mg/m^2 by intravenous bolus injection daily together with 20 mg/m^2 leucovorin for 5 days , followed by 3 weeks off. A second therapeutic regimen may consist of administering 200 to 220 mg/m^2 5-FU by continuous infusion over 24 hours once a week. A third therapeutic regimen consists of shorter, intermittent infusions of 5-FU from between 24 to 120 hours, every week, two weeks, three weeks or four weeks at dosages of 600 mg/m^2 to 2500 mg/m^2 per 24 hours. One skilled in the art will also appreciate that 5-FU and its variants can be used in combination therapies with a variety of other traditional chemotherapeutic drugs.

An exemplary therapeutic regimen for raltitrexed (Tomudex®) is administration at 3 mg/m^2 once every 3 weeks by bolus injection.

An exemplary regimen for pemetrexed is administration at 500mg/m2 once every 3 weeks. Pemetrexed may be used in this regimen alone or in combination with cisplatin. Examples of additional supportive drugs that could be included in the above regimen include: folic acid daily at 0.4mg, Vitamin B12 at 1000 micrograms every 9 weeks. Dexamethazone may also be included as a supportive drug.

Other chemotherapeutic agents contemplated by the present invention include those which may be applicable to a range of cancers, such as doxorubicin, capecitabine, mitoxantrone, irinotecan (CPT-11), as well as those that are suited to the treatment of a specific cancer.

Examples of chemotherapeutic agents suitable for the treatment of breast cancer include, but are not limited to, capecitabine, cyclophosphamide, ifosfamide, cisplatin, carboplatin, 5-fluorouracil (5-FU), taxol, taxanes such as paclitaxel and docetaxel and various anthracyclines, such as doxorubicin and epi-doxorubicin (also known as epirubicin). Combination therapies using standard cancer chemotherapeutics may also be used in conjunction with the antisense oligonucleotides and are also well known in

the art, for example, the combination of epirubicin with paclitaxel or docetaxel, or the combination of doxorubicin or epirubicin with cyclophosphamide, which are used for breast cancer treatments. Polychemotherapeutic regimens are also useful and may consist, for example, of doxorubicin/cyclophosphamide/5-fluorouracil or cyclophosphamide/epirubicin/5-fluorouracil. Many of the above chemotherapeutics and combinations thereof are useful in the treatment of a variety of solid tumours.

Cyclophosphamide, mitoxantrone and estramustine are known to be suitable for the treatment of prostate cancer. Cyclophosphamide, vincristine, doxorubicin and etoposide are used in the treatment of small cell lung cancer, as are combinations of etoposide with either cisplatin or carboplatin. In the treatment of stomach or oesophageal cancer, combinations of doxorubicin or epirubicin with cisplatin and 5-fluorouracil are useful. For colorectal cancer, CPT-11 alone or in combination with 5-fluorouracil-based drugs, or oxaliplatin alone or in combination with 5-fluorouracil-based drugs can be used. Oxaliplatin may also be used in combination with capecitabine.

Other examples include the combination of cyclophosphamide, doxorubicin, vincristine and prednisone in the treatment of non-Hodgkin's lymphoma; the combination of doxorubicin, bleomycin, vinblastine and DTIC in the treatment of Hodgkin's disease and the combination of cisplatin or carboplatin with any one or a combination of gemcitabine, paclitaxel, docetaxel, vinorelbine or etoposide in the treatment of non-small cell lung cancer. Pemetrexed alone is also a proven effective drug in the treatment of non-small cell lung cancer. Other suitable chemotherapeutic agents include, but are not limited to, mitomycin C, vinblastine, IL-2, novantrone, DTIC and hydroxyurea.

One embodiment of the present invention contemplates the use of the antisense oligonucleotides as "sensitizing agents," or "chemopetentiators," which selectively inhibit the growth of cancer cells. In this case, the antisense oligonucleotides alone does not have a cytotoxic effect on the cancer cell, but provides a means of weakening the cancer cells, and thereby facilitates the benefit from conventional anti-cancer therapeutics.

CLINICAL TRIALS IN CANCER PATIENTS

One skilled in the art will appreciate that, for the treatment of human patients, the antisense oligonucleotides, alone or in combination with one or more chemotherapeutic agents, should be tested in Clinical Trials in order to further evaluate their efficacy in the treatment of cancer and to obtain regulatory approval for therapeutic use. As is known in the art, clinical trials progress through phases of testing, which are identified as Phases I, II, III, and IV.

Initially the antisense oligonucleotides will be evaluated in a Phase I trial. Typically Phase I trials are used to determine the best mode of administration (for example, by pill or by injection), the frequency of administration, and the toxicity for the compounds. Phase I studies frequently include laboratory tests, such as blood tests and biopsies, to evaluate the effects of a compound in the body of the patient. For a Phase I trial, a small group of cancer patients are treated with a specific dose of the antisense oligonucleotide(s). During the trial, the dose is typically increased group by group in order to determine the maximum tolerated dose (MTD) and the dose-limiting toxicities (DLT) associated with the compound. This process determines an appropriate dose to use in a subsequent Phase II trial.

A Phase II trial can be conducted to evaluate further the effectiveness and safety of the antisense oligonucleotides. In Phase II trials, the antisense oligonucleotide is administered to groups of patients with either one specific type of cancer or with related cancers, using the dosage found to be effective in Phase I trials.

Phase III trials focus on determining how a compound compares to the standard, or most widely accepted, treatment. In Phase III trials, patients are randomly assigned to one of two or more "arms". In a trial with two arms, for example, one arm will receive the standard treatment (control group) and the other arm will receive treatment with the antisense oligonucleotide (investigational group).

Phase IV trials are used to further evaluate the long-term safety and effectiveness of a compound. Phase IV trials are less common than Phase I, II and III trials and will take place after the antisense oligonucleotide has been approved for standard use.

Eligibility of Patients for Clinical Trials

Participant eligibility criteria can range from general (for example, age, sex, type of cancer) to specific (for example, type and number of prior treatments, tumour characteristics, blood cell counts, organ function). Eligibility criteria may also vary with trial phase. For example, in Phase I and II trials, the criteria often exclude patients who may be at risk from the investigational treatment because of abnormal organ function or other factors. In Phase II and III trials additional criteria are often included regarding disease type and stage, and number and type of prior treatments.

Phase I cancer trials usually comprise 15 to 30 participants for whom other treatment options have not been effective. Phase II trials typically comprise up to 100 participants who have already received chemotherapy, surgery, or radiation treatment, but for whom the treatment has not been effective. Participation in Phase II trials is often restricted based on the previous treatment received. Phase III trials usually comprise hundreds to thousands of participants. This large number of participants is necessary in order to determine whether there are true differences between the effectiveness of the antisense oligonucleotides and the standard treatment. Phase III may comprise patients ranging from those newly diagnosed with cancer to those with extensive disease in order to cover the disease continuum.

One skilled in the art will appreciate that clinical trials should be designed to be as inclusive as possible without making the study population too diverse to determine whether the treatment might be as effective on a more narrowly defined population. The more diverse the population included in the trial, the more applicable the results could be to the general population, particularly in Phase III trials. Selection of appropriate participants in each phase of clinical trial is considered to be within the ordinary skills of a worker in the art.

Assessment of patients prior to treatment

Prior to commencement of the study, several measures known in the art can be used to first classify the patients. Patients can first be assessed, for example, using the Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) scale or the Karnofsky Performance Status (KPS) scale, both of which are widely accepted

standards for the assessment of the progression of a patient's disease as measured by functional impairment in the patient.

Patients' overall quality of life can be assessed, for example, using the McGill Quality of Life Questionnaire (MQOL) (Cohen *et al* (1995) *Palliative Medicine* **9**: 207-219). The MQOL measures physical symptoms; physical, psychological and existential well-being; support; and overall quality of life. To assess symptoms such as nausea, mood, appetite, insomnia, mobility and fatigue the Symptom Distress Scale (SDS) developed by McCorkle and Young ((1978) *Cancer Nursing* **1**: 373-378) can be used.

Patients can also be classified according to the type and/or stage of their disease and/or by tumour size.

Administration of the antisense oligonucleotides of the present invention in Clinical Trials

The antisense oligonucleotide is typically administered to the trial participants parenterally. In one embodiment, the antisense oligonucleotide is administered by intravenous infusion. Methods of administering drugs by intravenous infusion are known in the art. Usually intravenous infusion takes place over a certain time period, for example, over the course of 60 minutes. In other embodiments of the invention, for example, for the treatment of patients with mesothelioma, the antisense oligonucleotide is administered intracavitorially, *i.e.* by intrapleural, intraperitoneal or intrapericardial infusion.

Monitoring of Patient Outcome

The endpoint of a clinical trial is a measurable outcome that indicates the effectiveness of a treatment under evaluation. The endpoint is established prior to the commencement of the trial and will vary depending on the type and phase of the clinical trial. Examples of endpoints include, for example, tumour response rate – the proportion of trial participants whose tumour was reduced in size by a specific amount, usually described as a percentage; disease-free survival – the amount of time a participant survives without cancer occurring or recurring, usually measured in months; overall survival – the amount of time a participant lives, typically measured

from the beginning of the clinical trial until the time of death. For advanced and/or metastatic cancers, disease stabilisation – the proportion of trial participants whose disease has stabilised, for example, whose tumour(s) has ceased to grow and/or metastasise, can be used as an endpoint. Other endpoints include toxicity and quality of life.

Tumour response rate is a typical endpoint in Phase II trials. However, even if a treatment reduces the size of a participant's tumour and lengthens the period of disease-free survival, it may not lengthen overall survival. In such a case, side effects and failure to extend overall survival might outweigh the benefit of longer disease-free survival. Alternatively, the participant's improved quality of life during the tumour-free interval might outweigh other factors. Thus, because tumour response rates are often temporary and may not translate into long-term survival benefits for the participant, response rate is a reasonable measure of a treatment's effectiveness in a Phase II trial, whereas participant survival and quality of life are typically used as endpoints in a Phase III trial.

PHARMACEUTICAL KITS

The present invention additionally provides for therapeutic kits containing the antisense oligonucleotide and optionally one or more chemotherapeutic agents in pharmaceutical compositions for use in the treatment of cancer. Individual components of the kit would be packaged in separate containers and, associated with such containers, can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

When the components of the kit are provided in one or more liquid solutions, the liquid solution can be an aqueous solution, for example a sterile aqueous solution. In this case the container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the composition may be administered to a patient.

The components of the kit may also be provided in dried or lyophilised form and the kit can additionally contain a suitable solvent for reconstitution of the lyophilised components. Irrespective of the number or type of containers, the kits of the invention also may comprise an instrument for assisting with the administration of the composition to a patient. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

Antisense oligonucleotides

SEQ ID NO:1 as referred to throughout the Examples is a fully phosphorothioated ODN with 2'-methoxy-ethoxy modification on the 6 nucleotides at both the 5'- and 3'-ends, unless otherwise indicated.

The scrambled control ODN (SEQ ID NO:7): 5'-ATGCGCCAACGGTTCCTAAA-3' has the same base composition as ODN SEQ ID NO:1, in random order, and is not complementary to any region of human TS mRNA.

EXAMPLE 1: Effect of TS antisense oligonucleotide treatment on tumour cell proliferation

HeLa, MCF-7, HT-29, MSTO-211H, NCI-H2052, and NCI-H28 cell lines were plated at a density of 100,000 cells/flask (T-25) and were treated the following day with ODNs (10, 25, 50 or 100 nM) using LipofectAmine 2000 (Invitrogen) as a transfection agent. Cells were counted using a Coulter electronic particle counter after four days. Cell proliferation in the presence of TS antisense SEQ ID NO:1 is shown relative to proliferation in control ODN (SEQ ID NO:7) treated flasks.

Figure 2 shows that three mesothelioma-derived cell lines, NCI-H28, MSTO-211H and NCI-H2052, are exquisitely sensitive to TS antisense ODN treatment. Inhibition of proliferation of the breast tumour-derived MCF-7 and ovarian carcinoma OV-90 cells by TS antisense SEQ ID NO:1 was also observed.

EXAMPLE 2: Effect of TS antisense oligonucleotide treatment on TS mRNA levels in tumour cells

HeLa, HT-29 and MCF-7 cells were plated in T75 flasks at a density of 10^6 cells per flask. After attachment, the cells were treated with TS antisense SEQ ID NO:1 or scrambled control SEQ ID NO:7 (100 nM) mixed with LipofectAmine 2000 (1 μg/ml). After 24, 36, or 48 hours (as indicated), RNA was extracted using Trizol (Invitrogen). The RNA was reverse-transcribed and cDNA used as a template for PCR using specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or TS, as previously described (Berg et al. (2001) *J Pharmacol Exp Ther* **298**:477-484; Berg et al. (2003) *Cancer Gene Therapy* **10**:278-286). The abundance of GAPDH mRNA was unchanged by ODN treatment, while levels of TS mRNA in each cell line were specifically reduced by SEQ ID NO:1 treatment relative to control SEQ ID NO:7 treatment (Figure 3).

EXAMPLE 3: Antisense ODN delivery into tumour cell lines as monitored by *in situ* hybridization

An *in situ* hybridization assay to quantitate the effectiveness of antisense ODN delivery to tumour cells was developed. Human HeLa, MCF7 and HT-29 tumour cells were transfected with TS antisense ODNs using cationic lipid and fixed at various times with paraformaldehyde. *In situ* hybridization of biotinylated complementary ODNs was detected using streptavidin-horseradish peroxidase followed by diaminobenzidine.

In more detail, HeLa cells were treated with TS antisense SEQ ID NO:1 or scrambled control SEQ ID NO:7 in the presence of LipofectAmine 2000 (1 μg/ml) and fixed with paraformaldehyde. Biotin-labelled complementary ODNs (c83 and c32) were used as *in situ* hybridization probes, detected with horseradish peroxidase-conjugated

avidin and DAB as a substrate. Intense staining suggests mainly nuclear localisation of internalised ODNs (Figure 4A). Quantitation of stained cells are shown in Figure 4 for HeLa cells treated with 100 nM ODN for various times (Figure 4B) or treated for 24 hours with various ODN concentrations (Figure 4C). Following treatment for 6, 24, 36 and 48 hours with ODN (25, 50 or 100 nM), 83 to 90% of HeLa cells were intensely labelled. In accord with previous results showing recovery of TS mRNA and protein levels at later times, only 29% of cells fixed 72 hours after ODN exposure were stained.

ODN delivery into tumour cells was compared in HeLa, HT-29, and MCF-7 tumour cell lines. Uptake of antisense ODN into MCF-7 breast carcinoma cells was comparable to HeLa cells, and RT-PCR analysis indicated similar reductions in TS mRNA levels in these two cell lines. In contrast, only 46% of HT-29 colon carcinoma cells were labelled using the *in situ* hybridization technique, and ODN-mediated TS mRNA downregulation and ODN-induced inhibition of cell proliferation were also less effective in HT-29 compared with HeLa cells.

EXAMPLE 4: Effect of treatment with TS antisense ODN in combination with TS-targeting chemotherapeutic agents on HT-29 cells

HT-29 cells were plated in T25 flasks, treated with SEQ ID NO:1 or SEQ ID NO:7 (50 nM) and various concentrations of raltitrexed (RTX) or 5-fluorouracil deoxyribonucleotide (5-FudR). Cell proliferation was measured by counting cells after 4 days. The results shown in Figure 6 indicate increased inhibition of HT-29 proliferation by a combination of SEQ ID NO:1 and chemotherapeutic compared to control SEQ ID NO:7 and chemotherapeutic.

EXAMPLE 5: Effect of TS antisense ODN treatment on tumour cell proliferation

To measure the effects of ODNs on tumour cell proliferation, MSTO-211H, NCI-H28, NCI-H2052, MCF-7, and OV-90 cells were plated at a density of 1 X 10^5 per 25-cm^2 flask. After 24 or 48 hours, ODNs were mixed with Lipofectamine 2000 (LFA, Invitrogen) for 15 minutes in serum-free medium. Additional medium and FBS

were added to achieve the final concentrations of ODN indicated in Figure 7. Final concentrations of LFA ranged from 0.1 to 0.5 μg/ml to maintain a constant ODN:lipid ratio. The cell culture medium was replaced with 2 ml of the ODN:lipid mixture, and cells incubated for 4 hours, after which an additional 2 ml of medium with FBS was added. Cells in 3 flasks were counted using an electronic particle counter (Beckman Coulter, Hialeah, FL) at the time of treatment and after three days of culture. Cell proliferation in the presence of TS antisense SEQ ID NO:1 or control ODN (SEQ ID NO:7) is shown relative to proliferation in the presence of LFA alone.

Figure 7 shows that three mesothelioma-derived cell lines, NCI-H28, MSTO-211H and NCI-H2052, are exquisitely sensitive to TS antisense ODN (SEQ ID NO:1) treatment. Inhibition of proliferation of the breast tumour-derived MCF-7 and ovarian carcinoma OV-90 cells by TS antisense ODN SEQ ID NO:1 was also observed.

EXAMPLE 6: Effect of TS antisense oligonucleotide treatment on TS mRNA levels in mesothelioma cells

Mesothelioma cells were treated with TS antisense ODN SEQ ID NO:1 or control ODN SEQ ID NO:7 (10, 25 and 50 ng) mixed with LFA as described in Example 5. After 24 hours, RNA was isolated using Trizol (Invitrogen). RNA (2 ug) was reverse-transcribed using SuperScript II. Two to 5 % of the resulting cDNA was amplified by PCR using primers specific for GAPDH or TS. PCR products were separated on a 1.75% agarose gel stained with ethidium bromide. Image Master VDS gel documentation system and Image Quant software were used to quantitate staining intensity.

A representative example of RT-PCR products from 3 replicates of mesothelioma 211H cells treated with TS antisense ODN SEQ ID NO:1 or control ODN SEQ ID NO:7 is shown in Figure 8. Figure 9A-C illustrates the quantitation of RT-PCR products from mesothelioma 211H, 2052, and H28 cells treated with TS antisense ODN SEQ ID NO:1 compared to control ODN SEQ ID NO:7 relative to GAPDH PCR products. The mean plus standard deviation (n=3) is shown. Figures 8 & 9 show

that the levels of TS mRNA in each cell line were specifically reduced by SEQ ID NO:1 treatment relative to control SEQ ID NO:7 treatment.

EXAMPLE 7: Effect of treatment with TS antisense ODN in combination with TS-targeting chemotherapeutic agents on mesothelioma cell lines

Tumour cells were first treated with pemetrexed (Alimta®), 5-FUdR, or gemcitabine alone at various concentrations to determine the IC_{50} in a proliferation assay, using cell counting on day 0 and day 3.

Mesothelioma and OV-90 cells were pretreated with TS antisense ODN SEQ ID NO:1 plus control ODN SEQ ID NO:7 (5 nM each) or control ODN SEQ ID NO:7 (10 nM) with 0.1 μg/ml LFA for 4 hours as described in Example 5. MCF-7 cells were pretreated with 25 nM SEQ ID NO:7 or SEQ ID NO:1 (mixed with 0.25 ug/ml LFA). After the 4-hour incubation, 2 ml of medium with various concentrations of Alimta®, 5 -FUdR or gemcitabine was added to achieve the final drug concentrations indicated. Cells were counted at the time of drug treatment and 3 days later, as described in Example 5.

Figure 10 illustrates the effect of TS antisense ODN SEQ ID NO:1 in combination with Alimta® on tumour cell proliferation. The mean IC_{50} for Alimta® in the three mesothelioma cell lines was 22.5nM (+/- 4.3nM). Combined treatment with TS antisense ODN (5nM) reduced the Alimta® IC_{50} by fourfold to 5nM (+/- 2.5nM) in a greater than additive fashion and thus potentiates Alimta® cytotoxicity. Control scrambled ODN SEQ ID NO:7 had no effect on the IC_{50}, alone or in combination with Alimta®. The ability of TS antisense ODN SEQ ID NO:1 to potentiate the cytotoxic effect of Alimta® in MCF-7 cells and OV-90 cells was not as pronounced.

Figure 11 illustrates that TS antisense ODN SEQ ID NO:1 also potentiates the cytotoxicity of 5-FUdR in mesothelioma cells, and to a lesser extent in MCF-7 cells. Cytotoxicity of gemcitabine in mesothelioma cells was relatively unaffected by TS antisense ODN SEQ ID NO:1 (Figure 12).

The disclosure of all patents, publications, including published patent applications, and database entries referenced in this specification are specifically incorporated by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be incorporated by reference.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.